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Thymoquinone reduces kidney damage in apolipoprotein E-deficient mice fed a high-cholesterol diet

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Hypercholesterolemia is a well-established risk factor for kidney injury that can lead to chronic kidney disease (CKD). Thymoquinone (TQ) is one of the most active ingredients in *Nigella Sativa* seeds. It has a variety of beneficial properties including anti-oxidative and anti-inflammatory activities. TQ exerts positive effects on DOX-induced nephropathy and ischaemia-reperfusion induced kidney injury in rats. The goal of this study was to investigate the possible protective effects of thymoquinone against kidney injury in apolipoprotein E-knockout (ApoE^{-/-}) mice. Eight-week-old male ApoE^{-/-} mice were randomly divided into the following 3 groups: a control group fed a normal diet (ND group), a group fed a high-cholesterol diet (HD group) and a group fed HD mixed with thymoquinone (HD + TQ group). All groups were given the diet for 8 weeks. Metabolic characteristics including total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-c) were lower in ApoE^{-/-} HD + TQ mice than in ApoE^{-/-} HD mice. Oil-red O staining revealed excessive lipid deposition in the kidneys of ApoE^{-/-} HD mice; however, it was significantly suppressed in the ApoE^{-/-} HD + TQ mice. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) gene and protein expression was lower in the kidney tissues of ApoE^{-/-} HD + TQ mice than those of ApoE^{-/-} HD mice. Furthermore, macrophages and pro-inflammatory cytokines were lower in the kidney tissues of ApoE^{-/-} HD + TQ mice than in the ApoE^{-/-} HD mice. These results indicate that thymoquinone may be a potential therapeutic agent for kidney damage from hypercholesterolemia.

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Introduction

ApoE^{-/-} mice are a well-accepted model of hypercholesterolemia, and they have been extensively used to study the effects of this disease on atherosclerosis and renal injury.¹ One of our recent studies showed that in ApoE^{-/-} mice, dyslipidaemia-related kidney injury is associated with marked pathological alterations, including lipid deposition, pro-inflammatory gene expression and macrophage accumulation.² Increasing evidence has shown that lipid accumulation in the kidney contributes to the progression of chronic kidney disease (CKD).^{3,4} This process is enhanced in the presence of elevated levels of plasma lipids. Macrophages phagocytose oxidized

lipids and transition to foam cells. Macrophage-derived foam cells release cytokines that recruit more macrophages to lesions and influence lipid deposition.⁵ Therefore, similar pathogenic mechanisms may contribute to the progression of atherosclerosis and chronic kidney disease. However, the underlying pathophysiological mechanisms of the relationship between hypercholesterolaemia and kidney injury are not yet fully understood.

Recently, there has been a growing interest in using natural phytochemical compounds as treatment alternatives in several conditions including renal diseases. Indeed, it has been estimated that at least 25% of the drugs used over the past few decades have been directly derived from plants and approximately 25% are chemically altered natural products.⁶ Thymoquinone is one of these compounds. It is the main active ingredient of *Nigella sativa*, commonly known as black cumin or black seed, an annual flowering plant native to some areas such as the Mediterranean countries.⁷ Since its first extraction in 1963,⁸ thymoquinone has been shown to act as a potent free radical and superoxide scavenger.^{9–11} In addition, it has been shown to have anti-inflammatory properties *in vivo* and *in vitro* studies.^{12,13} It has also been shown to exert positive effects on DOX-induced nephropathy and ischaemia-reperfusion-induced

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kidney injury in rats.^{14,15} However, the function of thymoquinone in hypercholesterolemia-induced renal injury is unclear.

Materials and methods

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Dalian Medical University and approved by the Animal Ethics Committee of the First Affiliated Hospital of Dalian Medical University. ApoE^{−/−} mice were purchased from Beijing Vital River Lab animal technology Co., Ltd. (Beijing, China). All mice were housed in a room with a 12:12 hour light–dark cycle at 24 °C. At 8 weeks old, the male mice were randomly divided into the following three groups: ApoE^{−/−} mice fed a normal diet (*n* = 7), a high-cholesterol diet (*n* = 7), or thymoquinone by gavaged (25 mg per kg per day; Sigma-Aldrich, St. Louis, MO, USA) + a high-cholesterol diet (*n* = 7). The high-cholesterol diet contained 1.5% cholesterol and 15% fat. The experimental diet was purchased from the Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). Each group of mice were fed the corresponding diet for 8 weeks. Blood samples were obtained from the inferior vena cava and collected in serum tubes; the samples were then stored at −80 °C until use. Coronal sections of kidneys were fixed in 10% formalin and then embedded in paraffin for histological evaluation or embedded in OCT compound (Torrance, CA, USA) and stored at −80 °C for Oil-red O staining. The remainder of the kidney was snap-frozen in liquid nitrogen for mRNA or immunoblotting analysis. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The study was approved by the ethical committee of the First Affiliated Hospital of Dalian Medical University.

Biochemical measurements

Serum was obtained and stored at −80 °C. Total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-c) were measured using a Hitachi 7020 automatic analyser (Hitachi, Tokyo, Japan).

Morphologic analysis and immunohistochemistry

Kidney samples were collected and either fixed in 4% paraformaldehyde or snap frozen in liquid nitrogen. Samples were embedded in paraffin or OCT and were cut into slices using a microtome (Leica RM 2235 or Leica CM1850UV; Leica, Solms, Germany). The slices were then mounted onto glass slides, and histological examinations were performed. Frozen sections were used to evaluate lipid deposition using Oil-red O staining (Sigma, Santa Clara, CA, USA). Immunohistochemistry was performed using Histone Simple stain kits (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Briefly, paraffin-embedded sections were deparaffinised with xylene and then rehydrated in a descending series of ethanol. The sections were treated for 15 min with 3% H₂O₂ in methanol to inactivate endogenous peroxidases and were then incubated at room temperature for 1 hour with primary antibodies against CD68 (rabbit anti-CD68 antibody, 1:500; Abcam, England) or LOX-1 (rabbit anti-LOX-1 antibody, 1:200; Abcam). All sections

were observed under an Olympus B×40 upright light microscope (Olympus, Tokyo, Japan). We quantified positive area using NIH ImageJ software (<http://rsbweb.info.nih.gov/ij/>).¹⁶

RNA isolation and real-time PCR

Total RNA was isolated from glomeruli using ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA using a first-strand cDNA synthesis kit (SuperScript VILO cDNA Synthesis Kit; Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Gene expression was quantitatively analysed by real-time PCR using fluorescent SYBR Green technology (Light Cycler; Roche Molecular Biochemicals). β -actin cDNA was amplified and quantitated with each cDNA sample to normalize the relative amounts of target genes. The primer sequences are listed in Table 1.

Western blotting of kidney tissue

Proteins were extracted from renal cortical tissues using radio-immunoprecipitation assay buffer (P0013B; Beyotime, Shanghai, China). The samples were electrophoresed on 10% SDS-PAGE gels, and proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore, Billerica, MA, USA). The membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk and then incubated in primary antibody diluents (P0023A; Beyotime) and gently shaken overnight at 4 °C. Primary antibodies against LOX-1 (rabbit anti-LOX-1 antibody, 1:250; Abcam), phospho-ERK (rabbit anti-phospho-ERK, 1:1000; Cell Signalling Technology), and β -actin (1:1000; Cell Signalling Technology) were used. The membranes were then incubated with the secondary antibody (anti-rabbit Ig-G, 1:1000; Cell Signalling Technology) for 1 hour. This analysis was independently performed three times. The protein levels are expressed as protein/ β -actin ratios to minimize loading differences. The relative signal intensity was quantified using NIH ImageJ software.

Table 1 Primer oligonucleotide sequences^a

Gene	Primers
LOX-1	F:5'-CAAAGTCTCCCAACCAACCTGCAA-3' R:5'-ACATCCTGTCTTTCATGCGGCAAC-3'
SRA1	F:5'-GTAAAGGTGATGGGGGACA-3' R:5'-TCCCCTTCTCTCCCTTTTGT-3'
CD36	F:5'-CCTTAAAGGAATCCCCGTGT-3' R:5'-TGCAATTTGCCAATGTCTAGC-3'
ABCA1	F:5'-AGCCAGAAGGGAGTGTGACA-3' R:5'-CATGCCATCTGGGTAACCT-3'
TNF- α	F:5'-TCTCATGCACCACCATCAAGGACT-3' R:5'-ACCACTCTCCCTTTGCAGAACTCA-3'
IL-6	F:5'-TACCAGTTGCCCTTCTTGGGACTGA-3' R:5'-TAAGCCTCCGACTTGTGAAGTGGT-3'
β -actin	F:5'-CGATGCCCTGAGGGTCTTT-3' R:5'-TGGATGCCACAGGATTCAT-3'

^a Abbreviations: LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; SRA, scavenger receptor-A; ABCA1, ATP-binding cassette transporter A1; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.



Statistical analysis

All data were presented as the mean \pm SEM. Statistical analysis was performed using SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA). Intergroup variation was measured using one-way ANOVA and subsequent Tukey's test. The minimal level for statistical significance was set at $P < 0.05$.

Results

Metabolic characteristics

The metabolic characteristics of ApoE^{-/-} mice after 8 weeks of dietary treatment are summarized in Table 2. The HD group showed a marked increase in total cholesterol and non-high-density lipoprotein cholesterol levels in ApoE^{-/-} mice, but these

parameters were significantly decreased in the HD + TQ group. There was no difference between the HD + TQ and ND groups. Kidney/body weight ratio did not differ among the three groups.

Thymoquinone reduced renal lipid accumulation in ApoE^{-/-} mice in the HD group

We used Oil-red O staining to evaluate renal lipid accumulation. We detected increased lipid retention in the kidneys of ApoE^{-/-} mice in the HD group. Interestingly, the mice in the HD + TQ group displayed markedly reduced renal lipid deposition compared with the ApoE^{-/-} mice despite the consumption of HD (Fig. 1A and B).

Table 2 Metabolic data from the three groups after 8 weeks of different treatment^a

	ApoE ^{-/-} ND <i>n</i> = 7	ApoE ^{-/-} HD <i>n</i> = 6	ApoE ^{-/-} HD + TQ <i>n</i> = 7
Body weight (g)	27.32 \pm 1.52	29.47 \pm 1.95	28.63 \pm 1.83
Kidney/body weight ratio (mg g ⁻¹)	6.44 \pm 0.95	7.22 \pm 0.85	6.53 \pm 1.05
T-cholesterol (mg dl ⁻¹)	425.03 \pm 32.16*	1862.67 \pm 136.32	686.53 \pm 54.31*
Non-HDL-c (mg dl ⁻¹)	127.43 \pm 26.72*	679.32 \pm 85.16	218.47 \pm 23.96*

^a Abbreviations: TC, total cholesterol; HDL-c, high-density lipoprotein cholesterol. Data are means \pm SEM; *n* = 6–7 per group. * $P < 0.05$ vs. ApoE^{-/-} HD.

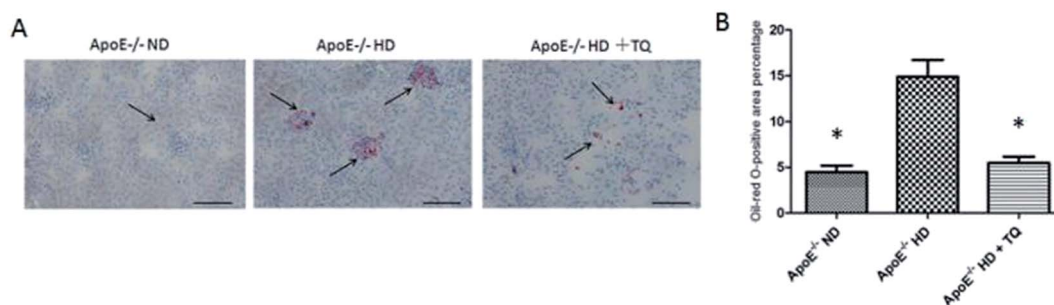


Fig. 1 Renal lipid accumulation in the three groups after 8 weeks with different diets. (A) Representative Oil-red O staining in the kidney tissue. Red: Oil-red O-positive cells, and blue: haematoxylin counterstaining. Scale bar = 200 μ m. (B) Bar graph showing oil red O-positive cells in the kidney tissues. Data are means \pm SEM; *n* = 4 in each group. * $P < 0.05$.

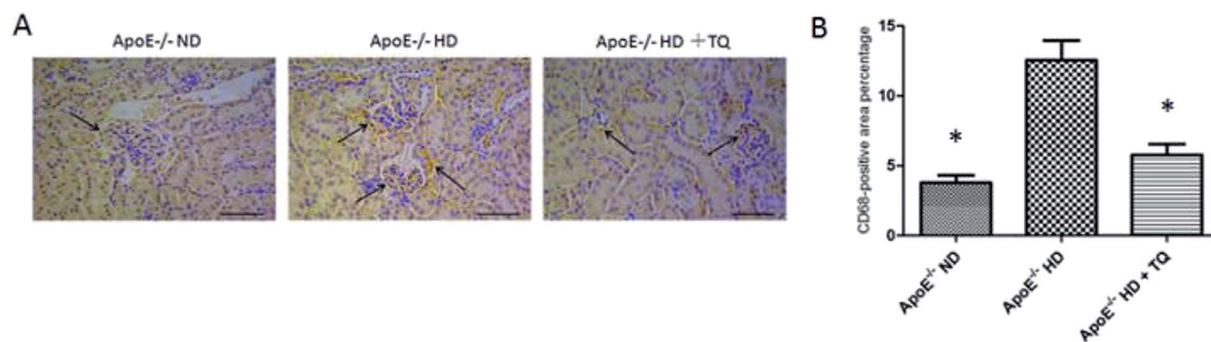


Fig. 2 CD68 expression in the kidney tissues of the three groups after 8 weeks with different diets. (A) Representative immunohistochemistry of CD68 expression in kidney tissues. Scale bar = 200 μ m. Arrows indicate positively stained cells. (B) Bar graph summarizing CD68 expression in kidney tissues. Each bar represents the mean \pm SEM; *n* = 4 in each group. * $P < 0.05$.



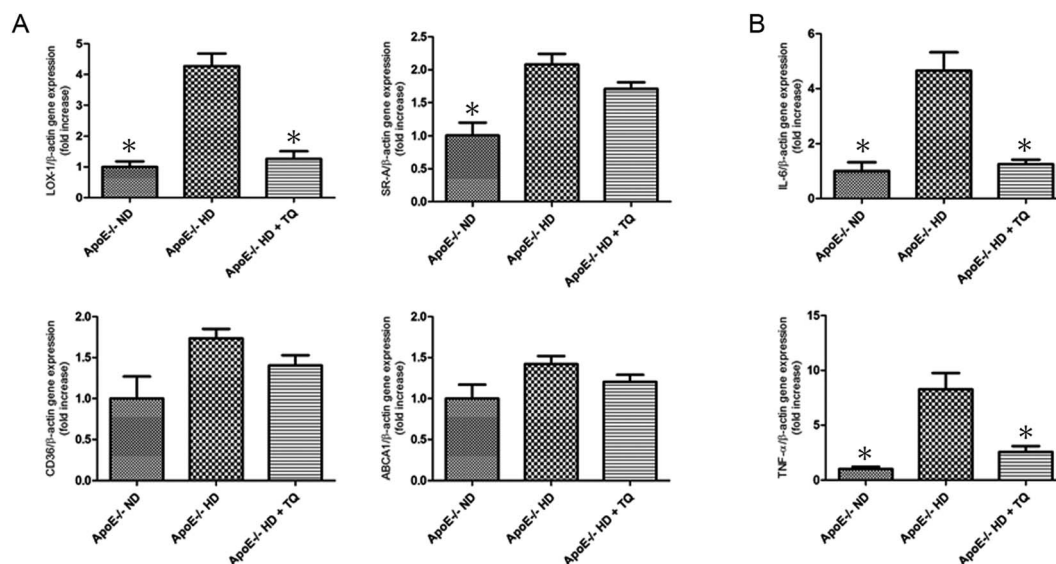


Fig. 3 Expression of scavenger receptors and pro-inflammatory genes in the kidney tissues of the three groups after 8 weeks with different diets. (A) Relative mRNA expression of LOX-1, SRA, CD36 and ABCA1 in the kidney tissues of the three groups after 8 weeks with different diets. (B) Relative mRNA expression of IL-6 and TNF- α in the kidney tissues of the three groups after 8 weeks with different diets. Data are given as the means \pm SEM; $n = 6$ in each group. * $P < 0.05$ vs. ApoE^{-/-} HD.

Thymoquinone reduced macrophages in the kidneys of ApoE^{-/-} mice in the HD group

To detect infiltrating macrophages, immunohistochemical analysis was performed against CD68 staining (Fig. 2A). The mice in the HD + TQ group showed markedly reduced CD68-positive staining in kidney tissues compared with ApoE^{-/-} HD mice (Fig. 2B). These results indicate that thymoquinone reduced macrophage infiltration in the ApoE^{-/-} HD mouse kidneys.

Thymoquinone reduced LOX-1 gene expression in the kidneys of ApoE^{-/-} mice fed HD

To investigate the mechanism of lipid accumulation in the kidneys, gene expression of relevant receptors and the ATP-binding cassette transporter A1 (ABCA1) was examined in kidney tissues using real-time PCR. LOX-1 gene expression was significantly increased in the kidney tissues of the HD group compared with that in the ND group. The increased expression

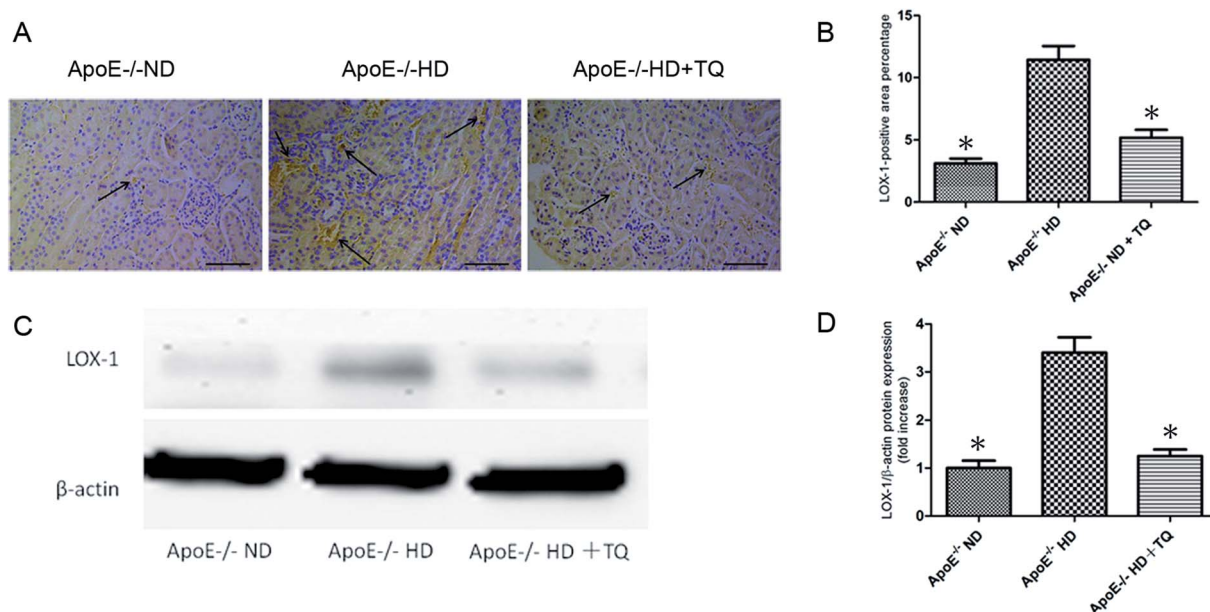


Fig. 4 LOX-1 expression in the kidney tissues of the three groups after 8 weeks with different diets. (A) Representative immunohistochemistry for LOX-1 in kidney tissues. Scale bar = 200 μ m. Arrows indicate positively stained cells. (B) Bar graph summarizing LOX-1 expression in kidney tissues. (C) Immunoblotting for LOX-1 in kidney tissues. (D) Bar graph showing quantification of LOX-1 protein expression. Data are given as the means \pm SEM; $n = 3-4$ in each group. * $P < 0.05$ vs. ApoE^{-/-} HD.



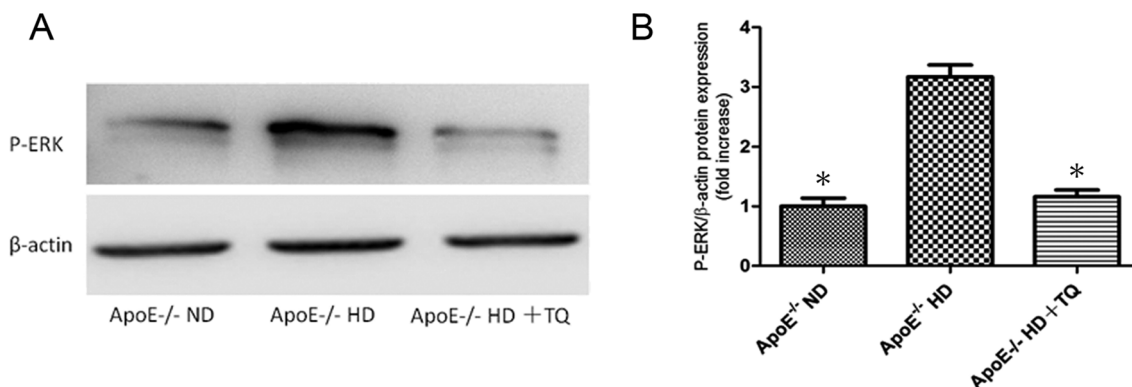


Fig. 5 Phospho-ERK protein expression in the kidney tissues of the three groups after 8 weeks with different diets. (A) Immunoblotting for phospho-ERK in kidney tissues. (B) Bar graph showing quantification of phospho-ERK protein expression. Data are given as the means \pm SEM; $n = 3$ in each group. * $P < 0.05$ vs. ApoE^{-/-} HD.

of LOX-1 was suppressed in the HD + TQ group. The expression of scavenger receptor-class A (SR-A) and CD36 was increased in the HD group compared with that in the ND group; however, the levels were similar to those for the HD + TQ group. The expression of ABCA1 did not differ among the three groups (Fig. 3A). These results suggest that LOX-1, SR-A, and CD36 influence lipid accumulation in the kidney tissues of ApoE^{-/-} HD mice. LOX-1, in particular, appears to be a critical factor for mitigating lipid accumulation in the kidney tissue of ApoE^{-/-} HD + TQ mice compared to that in the ApoE^{-/-} HD mice.

Thymoquinone reduced tumour necrosis factor (TNF)- α and interleukin (IL)-6 gene expression in the kidneys of ApoE^{-/-} mice fed HD

To examine the involvement of pro-inflammatory cytokines in the gene expression of hypercholesteremic kidney tissues, the gene expression of IL-6 and TNF- α was measured using real-time PCR (Fig. 3B). Both IL-6 and TNF- α were upregulated in the ApoE^{-/-} HD mice; however, this upregulation was attenuated in ApoE^{-/-} HD + TQ mice.

Thymoquinone reduced LOX-1 expression in the kidney tissues determined using immunohistochemistry and immunoblotting

To evaluate LOX-1 expression in the kidney tissues, LOX-1 immunostaining was performed (Fig. 4A). The HD + TQ group had markedly reduced LOX-1 expression in kidney tissues compared to the HD group (Fig. 4B). Immunoblotting was performed for LOX-1 protein (Fig. 4C). We found that the LOX-1 protein expression was significantly suppressed in the HD + TQ group compared with that in the HD group (Fig. 4D). These results indicate that thymoquinone reduced LOX-1 expression in ApoE^{-/-} HD mice.

Thymoquinone reduced the expression of phospho-ERK in the kidney tissues of ApoE^{-/-} HD mice

Protein kinases play a role in foam cell formation and lipid deposition,¹⁷ and immunoblotting for phospho-ERK protein

was performed (Fig. 5A). We found that phospho-ERK was significantly suppressed in the HD + TQ group compared to that in the HD group (Fig. 5B).

Discussion

This study demonstrates that thymoquinone has a protective effect against progressive lipid deposition, macrophage accumulation and pro-inflammatory cytokine mRNA content in the kidney elicited by hypercholesterolemia.

The cholesterol in ApoE^{-/-} mice is actually in LDL-C as well as in chylomicrons fractions. According to the metabolic characteristics, we found that TC and non-HDL-c were increased in the HD group compared with the ND group of ApoE^{-/-} mice. These results are agreement with reports by Daniel Kolbus.¹⁸ Interestingly, TC and non-HDL-c were significantly suppressed in the HD + TQ group compared with that in the HD group. These results indicate that thymoquinone influences cholesterol metabolism, Shafeeqe Ahmad *et al.* reported the same results and due to increased clearance rate of TG in hepatic β -VLDL secretion,^{19,20} however, further studies are needed to clarify the mechanisms.

Hypercholesterolemia is a major independent risk factor for kidney disease.²¹ In hypercholesterolemia, cellular lipid homeostasis involves the regulation of influx, synthesis, catabolism, and efflux of lipids. An imbalance in these processes can result in the conversion of macrophages, mesangial cells, and vascular smooth muscle cells into foam cells. This process is mediated by several independent factors, including SR-A, class B (CD36), and LOX-1, and it regulates the expression of the target gene ABCA1.^{22–24}

Thymoquinone is a potent phytochemical anti-oxidant due to its scavenging activity against several ROS including superoxide anions, hydroxyl radicals and singlet molecular oxygen, and thus it can antagonize the adverse effects resulting from elevated ROS levels in various disorders.^{25,26} It has been shown that thymoquinone and *tert*-butylhydroquinone (TBHQ), a structurally related synthetic compound, can robustly inhibit iron-dependent microsomal lipid peroxidation. The anti-



oxidative potential of thymoquinone may be related to the redox properties of its quinone moiety and its unrestricted ability to cross physiological barriers and access subcellular compartments, all of which help its radical scavenging effects.^{27,28}

These effects include the reduction in blood total cholesterol and lipid peroxidation levels during kidney injury.¹⁴ Thymoquinone administration has been shown to protect kidneys from oxidative damage caused by pyelonephritis. In pyelonephritis, SOD and CAT activity and MDA levels are significantly abnormal; and the deregulation of these factors is resolved in pyelonephritis treated with thymoquinone.²⁹

In our study, we analysed the gene expression of scavenger receptors including SR-A, CD36, and LOX-1. We found that LOX-1 gene expression was suppressed in the HD + TQ group. LOX-1 was originally identified in endothelial cells, and it is a 50 kDa type II membrane glycoprotein that contains a short N-terminal cytoplasmic domain, a single transmembrane domain, a short neck or stalk region, and an ox-LDL-binding C-terminal extracellular C-type lectin-like domain. On the cell surface, LOX-1 consists of 3 homodimers that are bound to ox-LDL, and it plays an important role in ox-LDL uptake and foam cell formation.^{30,31} In contrast, deletion of LOX-1 has been shown to reduce the uptake of oxidized LDL and inhibit atherosclerosis in mice fed a high-cholesterol diet.³² Therefore, suppression of LOX-1 expression in ApoE^{-/-} HD + TQ mice may reduce foam cell formation. Thymoquinone also reduced LOX-1 protein expression in the kidney tissues of ApoE^{-/-} HD mice. Protein kinases regulate foam cell formation and lipid deposition. As shown earlier, enhanced LOX-1 expression was attenuated by inhibitors of ERK, PKC and NF- κ B, indicating that the increased production of intracellular ROS and activation of the PKC/MAPK pathway are the initial signalling events in LOX-1 gene regulation.¹⁶ Our results show that phospho-ERK expression was significantly suppressed in the HD + TQ group compared with that in the HD group. It is speculated that thymoquinone regulates LOX-1 *via* the phospho-ERK pathway.

Pro-inflammatory genes (TNF- α and IL-6) have been reported to be expressed at high levels and contribute to kidney injury in hyperlipidaemia.^{33–35} In contrast, TNF- α and IL-6 were shown to induce LOX-1 upregulation in smooth muscle cells.³⁶ The present study shows that TNF- α and IL-6 gene expression is reduced in the HD + TQ group compared with that in the HD group. Attenuation of TNF- α and IL-6 expression may have also reduced LOX-1 expression in ApoE^{-/-} HD + TQ mice. It has been reported that thymoquinone reduces TNF- α and interleukin-6 in blood and tissues and protects tissues by reducing inflammation.^{12,13} However, another reported that reduction of p-ERK with reduction in inflammation such as TNF- α .³⁷

Kidney injury induced by hyperlipidaemia is usually associated with an increase in the number of macrophages. Macrophage-derived foam cells release cytokines that recruit more macrophages to lesions and influence lipid deposition.⁵ The marker CD68 identifies macrophages. CD68-positive cells have been found to infiltrate glomeruli and interstitial lesions.³⁸ As far as reported that LOX-1 increased with CD68 infiltration of atherosclerotic plaques in ApoE^{-/-} mice³⁹ and LOX-1 expression reduced with lower level of CD68 of high cholesterol diet

induced mice.⁴⁰ CD68 is a marker of pro-inflammatory M1, “classically activated” M1 macrophages produce proinflammatory cytokines such as TNF- α .⁴¹ In the present study, immunohistochemical staining with anti-CD68 antibody showed that CD68-positive cells were significantly increased in the HD group compared with that in the ND group of ApoE^{-/-} mice; however, mice in the HD + TQ group showed markedly reduced accumulation of CD68-positive cells in kidney tissues compared with mice in the HD group, similarly, with the TNF- α gene expression. This indicates that thymoquinone reduces macrophage accumulation and TNF- α gene expression in mice in the HD group.

In conclusion, our data establish that thymoquinone contributes to the mitigation of hypercholesterolaemic kidney injury as shown by the downregulation of LOX-1 and the suppression of foam cell formation, lipid deposition, and macrophage accumulation. These findings provide new insights into the role of thymoquinone in hypercholesterolemia-induced kidney injury and raise the possibility of a novel therapeutic intervention for treating the progression of chronic kidney disease.

Author contributions

Hongyang Liu designed this study; Guang Yang and Nan Li helped perform the experiments; Zuowei Pei, Yingshu Liu and Liyue Zhu analysed the data and interpreted the experimental results; Zuowei Pei prepared the figures and drafted the manuscript; and Hongyang Liu helped to revise the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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